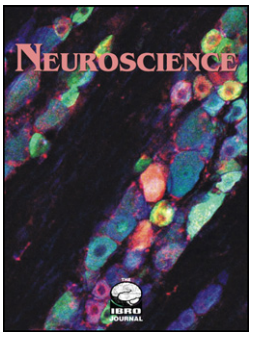

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Survival of Motor Neuron (SMN) protein over-expression prevents calpain mediated cleavage and activation of procaspase-3 in differentiated human SH-SY5Y cells

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List of abbreviations

SMA: Spinal muscular atrophy

SMN1: Survival of Motor Neuron 1

SMN: Survival of motor neuron protein

SMN1: Survival of Motor Neuron 1

GFP: Green Fluorescent Protein

DIV: On day *in vitro*

DMEM: Dulbecco's Modified Eagle Medium

FCS: foetal calf serum

MOI: multiplicity of infection

PBS: phosphate buffered saline

BDNF: brain-derived neurotrophic factor

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

PI3: phosphatidylinositol-3 kinase

H_2O_2: hydrogen peroxide

Abstract

Spinal muscular atrophy (SMA), a neurodegenerative disorder primarily affecting motor neurons, is the most common genetic cause of infant death. This incurable disease is caused by the absence of a functional *SMN1* gene and a reduction in full length SMN protein. In this study, a neuroprotective function of SMN was investigated in differentiated human SH-SY5Y cells using an adenoviral vector to over-express SMN protein. The pro-survival capacity of SMN was assessed in an Akt/PI3-kinase inhibition (LY294002) model, as well as an oxidative stress (hydrogen peroxide) and excitotoxic (glutamate) model. SMN over-expression in SH-SY5Y cells protected against Akt/PI3-kinase inhibition, but not oxidative stress, nor against excitotoxicity in rat cortical neurons. Western analysis of cell homogenates from SH-SY5Y cultures over-expressing SMN harvested pre- and post- Akt/PI3-kinase inhibition indicated that SMN protein inhibited caspase-3 activation via blockade of calpain mediated procaspase-3 cleavage. This study has revealed a novel anti-apoptotic function for the SMN protein in differentiated SH-SY5Y cells. Finally, the cell death model described herein will allow the assessment of future therapeutic agents or strategies aimed at increasing SMN protein levels.

Keywords: *SMN; anti-apoptotic; adenoviral; SMA; caspase-3.*

1.1 Introduction

With an incidence of 1 in 6,000 live births, spinal muscular atrophy (SMA), an autosomal recessive disorder characterised by progressive motor neuron loss and muscle weakness, is the leading genetic cause of infant mortality (Lefebvre et al., 1995; Crawford and Pardo, 1996; Feldkotter et al., 2002). Spinal muscular atrophy is caused by mutation(s)/deletion of the S*urvival of motor neuron 1* (*SMN1)* gene, which leads to a critical reduction in survival of motor neuron (SMN) protein levels. Humans uniquely possess a second centromeric *SMN* gene, termed *Survival of motor neuron 2* (*SMN2),* which is derived from a duplication and inversion event on chromosome 5 (Lefebvre et al., 1998; Rochette et al., 2001). The *SMN2* gene contains a synonymous base change in exon 7 (C→T) which forces exclusion of exon 7 from approximately 90% of pre-mRNA transcripts, resulting in the formation of a truncated and unstable protein (Lefebvre et al., 1995; Lorson and Androphy, 2000). The clinical phenotype and severity of SMA is categorised into four types, based on the age of onset, motor achievement and SMN2 copy number (Roberts et al., 1970; McAndrew et al., 1997).

Ubiquitously expressed throughout the CNS and particularly in the spinal cord, the SMN protein can localise to the nucleus of cells as part of structures known as gems (Liu and Dreyfuss 1996; Coovert et al., 1997; Lefebvre et al., 1997). In addition, the SMN protein, when localising to the cytoplasm and nucleus of cells plays an important role in the assembly of small nuclear ribonucleoproteins (snRNP's). However, whilst the latter function is well understood, SMN's role in motor neuron survival remains unclear (Rossoll et al., 2003). Regardless, evidence suggests that the loss of SMN's anti-apoptotic function is a contributing factor in SMA (Kerr et al., 2000).

Over-expression of SMN protein protects cells against nerve growth factor deprivation in rat PC12 cells, camptothecin induced apoptosis in human SMA patient fibroblasts and staurosporine induced death in mouse NSC34 cells (Vyas et al., 2002; Wang et al., 2005; Parker et at., 2008). In this study, adenoviral vectors were used to investigate the survival mechanisms of the SMN protein in both apoptotic and oxidative models in differentiated human SH-SY5Y neuroblastoma cells, and in an excitotoxic model in rat cortical neurons. For the first time, this study also compares the anti-apoptotic mechanisms used by SMN and Bcl-xL, another protein also implicated in SMA (Soler-Botija et al., 2003).

1.2 Experimental procedures

1.2.1 Cloning of human SMN cDNA and construction of adenoviral vectors

Total human RNA was isolated from HEK293 cells, reverse transcribed and amplified by PCR using a gene specific primer pair containing unique restriction sites (bold) and a Kozak sequence (underlined) as follows: - forward (*KpnI*) 5'**GGTACC**AGATCTGCCACCATGGCGATG-AGCAGCGG3' and reverse (*HindIII*) 5'**AAGCTT**TTAATTTAAGGAATGTGAGCACC3'. The resulting PCR products were gel purified, ligated into pGEM-Teasy (Promega, Madison, WI) and sequence verified. For subcloning, the SMN cDNA fragment was released by restriction enzyme digestion and ligated into the modified shuttle plasmid pRSV/WPRE/CMV:GFP (Boulos et al., 2006) to generate the vector: pRSV:SMN1/CMV:EGFP.

Recombinant adenoviruses were prepared according to the method of He et al. (1998), with some modifications (Boulos et al., 2006). Briefly, pShuttle plasmid DNA

(pRSV:SMN1/CMV:GFP) was linearized by *PmeI* digestion and introduced, by electroporation (Gene Pulser II, Biorad) into the *Escherichia coli* strain BJ5183 harboring the pAdeasy plasmid (Zeng et al., 2001). Recombinants were selected on media containing 50 μg/ml kanamycin, and their plasmid DNA analysed by *PacI* digestion. HEK293 cells grown to 90% confluency in 25cm^2 flasks were transfected with 3 μg of *PacI* linearized recombinant plasmid DNA using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Following the appearance of viral plaques (5-10 days), culture lysates were used for viral amplification in HEK293 cells. The SMN1 expressing adenoviral vector was designated AdRSV:SMN:CMV:GFP (AdRSV:SMN). Adenoviral particles were purified and concentrated from HEK293 cell lysates using the Adeno-X kit (BD Biosciences, San Jose, CA). Viral titres were determined by end-point dilution assay as indicated by enhanced GFP reporter expression. Vectors consisting of an adenovirus expressing only green fluorescent protein (GFP; AdRSV:Empty:CMV:GFP; AdRSV:Empty) and an adenovirus over-expressing Bcl-xL (AdRSV:Bcl-xL:CMV:GFP; AdRSV:Bcl-xL) have been described previously (Boulos et al., 2006).

1.2.2 Rat primary cortical neuronal cell cultures

All animal procedures were approved by the University of Western Australia Animal Ethics Committee. Establishment of cortical cultures was as previously described (Meloni et al, 2001). Briefly, cortical tissue from E18 to E19 Sprague-Dawley rats were dissociated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 1.3 mM L-cysteine, 0.9 mM $NaHCO_3$, 10 U/ml papain (Sigma, St. Louis, MO) and 50 U/ml DNaseI (Sigma) and washed in cold DMEM/10% horse serum. Culture 96-well plates were coated with poly-D-Lysine (50 μg/ml; 70-150K; Sigma) and incubated overnight at room

temperature. The poly-D-Lysine was removed and replaced with Neurobasal Media (containing 2% B27; 4% FCS; 1% horse serum; 62.5 µM glutamate; 25 µM 2-mercaptoethanol and 30 µg/ml penicillin). Neurons were plated at a density of 40,000 cells per well and cultures were maintained in a CO_2 incubator (5% CO_2; 95% air balance and 98% humidity) at 37^{o}C. On day *in vitro* (DIV) 4 half the media was removed and replaced with fresh NB/2% B27 (NB2°) containing the mitotic inhibitor cytosine arabinofuranoside. One third of the culture media was removed and replaced with fresh NB2° DIV 9, and the cultures were maintained at 37^{o}C in 5% CO_2.

1.2.3 Control fibroblasts and SMA patient fibroblasts

Control human fibroblasts (AGO6814; Coriell Institute, Camden, NJ) and SMA type I patient fibroblasts (GMO3813; Coriell Institute,) were maintained in DMEM containing penicillin (20 units/ml), streptomycin (20mg/ml) and foetal calf serum (FCS; 5%; heat-inactivated) and incubated at 37°C (5% CO_2).

1.2.4 SH-SY5Y cell propagation and differentiation

SH-SY5Y neuroblastoma cells were maintained in DMEM containing penicillin (20 units/ml), streptomycin (20mg/ml) and foetal calf serum (FCS; 5%; heat-inactivated) and incubated at 37°C (5% CO_2). To obtain differentiated SH-SY5Y cultures, cells were seeded into a 96 well plate (≈30,000 cells/well) in 100 µl of DMEM (5% FCS) containing all-trans retinoic acid (15 µM; Sigma). After 3 days, half the media was replaced with serum free DMEM and retinoic acid (15 µM). Five days after plating, half the media was replaced with serum free DMEM containing brain-derived neurotrophic factor (BDNF; 2 µM; Sigma), and maintained for a

further 3 days before use. Undifferentiated SH-SY5Y cells showed a rounded appearance with short processes, compared to retinoic acid treated SH-SY5Y cells which displayed a more neuronal-like morphology, with cells appearing triangular and showing longer processes (Encinas et al., 2000; Jamsa et al., 2004).

1.2.5 Adenoviral transduction of SH-SY5Y cells in 96 well plates

Adenovirus was diluted in serum free DMEM media containing BDNF (2 µM) and added to differentiating SH-SY5Y cultures 5 days after plating. To ensure uniform transduction of each virus in SH-SY5Y cultures (multiplicity of infection; MOI: 35-50), GFP reporter expression was initially quantitatively measured with a fluorescence plate reader (SPECTROstar; Omega) and subsequently routinely assessed by fluorescent imaging (Olympus IX70; Olympus DP70 digital camera). Cultures were used for experiments 3 days after viral transduction.

1.2.6 Adenoviral transduction of rat primary cortical neurons and fibroblasts in 96 well plates

Adenovirus was diluted in pre-conditioned media and added to cells in culture. To ensure uniform transduction of each virus in both cortical neurons and fibroblasts (MOI: 70-90), GFP reporter expression was routinely assessed by fluorescent imaging (Olympus IX70; Olympus DP70 digital camera). Cultures were used for experiments 3 days after viral transduction.

1.2.7 Immunohistochemistry of SMA patient fibroblasts

SMA type I patient fibroblasts (GMO3813; Coriell Institute, USA) grown on cover slips were fixed in ice cold 4% formalin in phosphate buffered saline (PBS) for 1 hour. Cover slips were rinsed in PBS-tween (PBS-T), blocked in 10% goat serum and exposed to an anti-SMN (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody diluted in PBS-T (1% goat serum) at 4°C overnight. Primary antibody was removed by washing in PBS prior to exposure with the secondary antibody (Alexa Fluor 488 goat anti-mouse, 1:400; Invitrogen) diluted in PBS-T at 4°C for 2 hours. Cover slips were rinsed with PBS, mounted onto slides, and viewed using fluorescence microscopy.

1.2.8 Cell injury models

1.2.8.1 Hydrogen peroxide oxidative stress model

To determine if the SMN protein could protect against oxidative stress, a hydrogen peroxide (H_2O_2) model was used. Bissonnette et al. (2004) has previously shown that in differentiated SH-SY5Y cultures exposed to 270 µM H_2O_2, cell viability was decreased by ≈50%. Differentiated SH-SY5Y cultures were treated with 300 µM H_2O_2 in serum-free DMEM media, and cell death was assessed 16-18 hours (37°C; 5% CO_2) after H_2O_2 addition.

1.2.8.2 Inhibition of pI3-kinase/akt cell signalling apoptosis model

To assess the potential anti-apoptotic function of the SMN protein, differentiated SH-SY5Y cultures were treated with a selective phosphatidylinositol-3 kinase (PI3) inhibitor, LY294002 (Sigma), which inhibits BDNF mediated pro-survival signalling resulting in a predominantly apoptotic cell death (Fujiwara et al., 2006). This model involved replacing

media in wells with serum-free DMEM media containing LY294002 (12.5-100 µM in DMSO), and incubating cultures for 16-18 hours (37°C; 5% CO_2) prior to cell death assessment.

1.2.8.3 Glutamate excitotoxicity model

Cultures were treated on DIV12 with 100 µM glutamate for 5 minutes. Media was removed and replaced with a 50% NB2°/balanced salt solution and incubated for 1 hour at 37^{o}C in 5% CO_2. Cell death was assessed using the LDH assay.

1.2.9 CytoTox 96® non-radioactive cytotoxic assay (LDH assay)

Cell death assessment was performed using the CytoTox 96® Non-Radioactive Cytotoxic Assay (LDH assay; Invitrogen). This assay which measures LDH released from dead cells, following the enzymatic (LDH) mediated conversion of the tetrazolium salt substrate into its red formazan product (detected spectrophotometrically at 490nm), was performed according to the manufacturers instructions. The amount of formazan product is proportional to the number of lysed/dead cells in culture.

1.2.10 Protein extraction from cells and western blotting

Proteins were extracted from SH-SY5Y cells using RIPA lysis buffer. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA), and equivalent amounts of protein (10 µg per lane) were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using pre-cast Bis-Tris gels (Invitrogen). For western blot analysis, proteins separated by SDS-PAGE were transferred to PVDF membranes. Membranes were briefly washed in PBS, blocked in PBS-Tween 20 (0.1%) containing

ovalbumin (1 mg/ml) for 1 hour and incubated in primary antibodies (Bcl-xL: 1:2000, BD Biosciences; SMN: 1:2500, Santa Cruz Biotechnology; Caspase-3: 1:2000, Santa Cruz Biotechnology; α-fodrin: 1:600, MP Biomedicals, Solon, OH) diluted in PBS-T (0.1%) plus ovalbumin (1 mg/ml) overnight at 4°C with gentle rocking. For protein detection, membranes were washed in PBS-T (0.1%), incubated with a HRP complexed secondary antibody (1:10000-1:25000 donkey anti-rabbit/sheep anti-mouse; GE Healthcare, Piscataway, NJ) for 1 hour at room temperature, washed in PBS, and visualised using ECL plus detection reagent (GE Healthcare). Quantification and protein band densitometry of western blots was undertaken using ImageJ (NIH) software.

1.2.11 Statistics

Statistical differences between experimental groups were determined by ANOVA, followed by post-hoc Fischer's PLSD test. $p < 0.05$ was considered statistically significant (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$). Unless otherwise stated, all experiments were conducted at least three times.

1.3 Results

1.3.1 Adenoviral mediated protein over-expression in differentiated SH-SY5Y cells and SMA patient fibroblasts

Transduction of differentiated SH-SY5Y cells was confirmed by fluorescence microscopy of GFP reporter expression (Fig 1A). Western blot analysis of lysates from differentiated SH-SY5Y cells transduced with AdRSV:SMN and AdRSV:Bcl-xL confirmed the over-expression of

SMN and Bcl-xL proteins respectively (Fig 1B). In addition, immunohistochemical analysis confirmed SMN over-expression by AdRSV:SMN in SMA patient fibroblasts (Fig 2). Over-expressed SMN protein appeared as cytoplasmic punctate structures consistent with previous studies (Gangwani et al., 2001).

1.3.2 SMN over-expression does not protect against hydrogen peroxide (oxidative stress)

AdRSV:SMN mediated over-expression of SMN in differentiated SH-SY5Y cultures did not protect cells from hydrogen peroxide exposure. By contrast, cultures transduced with the AdRSV:Bcl-xL vector exhibited reduced cell death following hydrogen peroxide exposure (Fig 3).

1.3.3 SMN over-expression does not protect against glutamate (excitotoxicty)

AdRSV:SMN mediated over-expression of SMN in rat primary cortical neurons did not protect cultures from excitotoxic injury. However, cultures over-expressing the Bcl-xL protein showed reduced cell death following glutamate exposure (Fig 4).

1.3.4 SMN over-expression protects against PI3-kinase/Akt inhibition (apoptosis)

A LY294002 dose response experiment using AdRSV:Empty vector transduced differentiated SH-SY5Y cultures revealed that a 25 µM dose resulted in ≈70% cell death (Fig 5), and thus, this concentration was used for subsequent experiments.

AdRSV:SMN transduction of differentiated SH-SY5Y cultures resulted in significantly reduced cell death following LY294002 exposure ($p < 0.005$; Fig 6). Similarly, AdRSV:Bcl-xL transduced

SH-SY5Y cultures also exhibited significantly reduced cell death following LY294002 exposure ($p < 0.0005$; Fig 6).

1.3.5 SMN protein reduces caspase-3 activity

The cytoskeletal protein, α-fodrin, is susceptible to cleavage by both caspase-3 and calpain. Therefore, a time course of caspase-3 and calpain activation based on α-fodrin cleavage following exposure to LY294002 was undertaken in differentiated SH-SY5Y cells. The time course revealed that the 120 and 145/150KDa α-fodrin degradation products significantly increased 2 hours after LY294002 exposure (Fig 7). Therefore, the 2 hour post LY294002 exposure time point was chosen to assess the effects of SMN over-expression on α-fodrin degradation in SH-SY5Y cells.

Differentiated SH-SY5Y cultures transduced with the AdRSV:SMN vector and exposed to LY294002 showed a significant reduction in α-fodrin 120 and 145/150KDa degradation products (Fig 8). Again, the effect was similar to that observed following transduction of SH-SY5Y cells with the vector, AdRSV:Bcl-xL (Fig 8). The cleavage products are representative of caspase activity (120, 145/150Kda products) and/or calpain activity (145/150Kda product), demonstrating that both SMN and Bcl-xL reduce caspase activity, and potentially calpain activity, following LY294002 exposure.

1.3.6 SMN protein prevents calpain-mediated cleavage of the caspase-3 prodomain

Activation of procaspase-3 (32kDa) requires cleavage of the 3kDa amino-terminal to form a pro-apoptotic p29 subunit (29kDa; McGinnis et al., 1999; Wolf et al., 1999). Western

analysis of caspase-3 protein from differentiated SH-SY5Y cells transduced with AdRSV:SMN showed no detectable caspase-3 p29 subunit, unlike AdRSV:Bcl-xL and AdRSV:Empty transduced SH-SY5Y cells (Fig 9).

Western analysis of SH-SY5Y cells 2 hours post LY294002 exposure, revealed that AdRSV:SMN transduced cells harbour reduced levels of the p29 subunit compared to AdRSV:Bcl-xL and AdRSV:Empty transduced cells (Fig 9). In contrast, the caspase-3 20kDa intermediate subunit, which is generated after caspase-9 cleavage of procaspase-3, showed no difference in levels between SH-SY5Y cells transduced with the different viral vectors (Fig 9).

1.4 Discussion

In SMA, a significant number of motor neurons in the spinal cord die by a process characteristic of apoptosis (Simic et al., 2000; Tsai et al., 2006; Simic et al., 2008). Furthermore, over-expression of SMN protein reduces cell death following apoptotic insults in rat primary cortical neurons, rat PC-12 cells and mouse NSC34 cells (Kerr et al., 2000; Vyas et al., 2002; Parker et al., 2008), however, to date, clarification of SMN's specific pro-survival mechanism(s) is lacking. In characterising a PI3-kinase/Akt inhibition apoptotic cell death model using differentiated SH-SY5Y human neuronal-like cells, we demonstrated that SMN protein over-expression reduces caspase-3 activation by preventing calpain mediated cleavage of the caspase-3 prodomain (Wolf et al., 1999). Our data thus supports and extends previous findings that SMN plays a role in modulating apoptosis and reducing caspase-3 activity (Kerr et al., 2000; Vyas et al., 2002; Wang et al., 2005; Parker et al., 2008).

The activation of caspase-3 (32KDa) involves a two stage processing event; the removal of its prodomain (3KDa) resulting in a 29KDa subunit (p29) and subsequent spontaneous proteolytic cleavage of p29 into large and small subunits (17/12kDa; Han et al., 1997; Deveraux et al., 1998; Meergans et al., 2000). SMN over-expression in untreated and dying SH-SY5Y cells resulted in the absence or reduction of the caspase-3 p29 subunit, indicating that prodomain removal was inhibited. Cleavage of the caspase-3 prodomain occurs via the action of calpain (Wolf et al., 1999). How SMN is able to block calpain cleavage of caspase-3 remains unclear. Interestingly, calpain also cleaves SMN protein, generating a 10kDa and 28kDa fragment, which may impart a pro-survival function (van Bergeijk et al., 2007; Walker et al., 2008). Alternatively, it is possible that SMN can block access to procaspase-3 cleavage in a manner similar to heat shock protein 27 (Hsp27), which is mutated in an axonal form of Charcot-Marie-Tooth disease (CMT2F), and like SMN, reduces caspase-3 activation and apoptosis (Pandey et al., 2000; Benn et al., 2002; Concannon et al., 2003; Reilly and Shy, 2009).

Bcl-xL is a well characterized potent anti-apoptotic member of the Bcl-2 family, which can protect against excitotoxic and oxidative injury (Xu et al., 1999; Dietz et al., 2007). Thus, as demonstrated in this study, Bcl-xL can also protect against non-apoptotic related cell death such as necrosis (Tsujimoto et al., 1997; Panickar et al., 2005). Hydrogen peroxide induced oxidative stress triggers apoptosis at lower concentrations and necrosis at higher concentrations (Skulachev, 2006). Therefore, the H_2O_2 concentration used in this study may cause predominantly necrotic cell death which explains why SMN over-expression was not

neuroprotective. This study also supports findings by others that SMN protein over-expression does not protect neuronal cultures against glutamate induced excitotoxicity (Cisterni et al., 2000). In our model, glutamate exposure causes calpain activation without caspase-3 activation (Meade et al., 2009). The lack of protection seen in the excitotoxic model is consistent with the notion that SMN can only protect against caspase-3 dependent apoptosis (Vyas et al., 2002; Wang et al., 2005; Parker et al., 2008).

Expression of Bcl-xL protein is reduced during fetal development in SMA patients (Soler-Botija et al., 2003). However, in our study, Bcl-xL over-expression did not reduce calpain mediated cleavage of caspase-3 p29 subunit levels in untreated and dying SH-SY5Y cells, suggesting it exerts its anti-apoptotic function through a different mechanism to SMN. Curiously, Bcl-xL over-expression can increase lifespan, improve motor function and reduce motor neuron degeneration in SMA mice (Tsai et al., 2008). Thus, our results suggest that treatments aimed at halting neuronal degeneration in SMA may benefit from the manipulation of both SMN and Bcl-xL survival pathways.

In summary, this study has shown that SMN over-expression reduces caspase-3 cleavage activation by specifically blocking calpain mediated cleavage of the caspase-3 prodomain. However, the data presented in this study is preliminary and requires further verification *in vivo* using a relevant animal model. In addition, we have established a novel apoptotic model of SMN functionality in a human neuronal cell line, which can be used to further elucidate the pro-survival mechanisms of the SMN protein, as well as a basis to select and test potential drug targets.

1.5 Acknowledgements

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Figure legends

Figure 1 Confirmation of adenoviral mediated transduction and expression in differentiated SH-SY5Y cells. A: GFP reporter expression in SH-SY5Y cells transduced with AdRSV:SMN, AdRSV:Empty and AdRSV:Bcl-xL, B: Western blot analysis of protein extracted from differentiated SH-SY5Y cells transduced with either AdRSV:Empty, AdRSV:SMN or AdRSV:Bscl-x_L. Expression of β-tubulin served as the loading control.

Figure 2 Immunofluorescence analysis of AdRSV:SMN transduced SMA I patient fibroblasts. A: Non-transduced SMA I patient fibroblasts probed with an anti-SMN antibody, B: SMN protein detected in AdRSV:SMN transduced SMA patient fibroblasts, C: GFP reporter expression in AdRSV:SMN transduced SMA I patient fibroblasts, D: Merged image overlapping GFP reporter expression and SMN localisation.

Figure 3 LDH assay comparing cell death between transduced differentiated SH-SY5Y cultures. Differentiated SH-SY5Y cultures transduced with AdRSV:SMN, AdRSV:Bcl-xL and AdRSV:Empty followed by hydrogen peroxide (300μM) treatment for 18h.

Figure 4 LDH assay comparing cell death between transduced primary rat cortical neurons. Primary cultures were transduced with AdRSV:SMN, AdRSV:Bcl-xL and AdRSV:Empty followed glutamate (100μM) treatment for 2h.

Figure 5 Dose-dependent induction of SH-SY5Y cell death by the PI3 kinase/Akt inhibitor, LY294002. LDH assay comparing cell death in AdRSV:Empty transduced differentiated SH-SY5Y cultures in response to increasing concentrations of LY294002 (12.5-100μM).

Figure 6 LDH assay comparing cell death in transduced differentiated SH-SY5Y cells. Measured LDH levels in AdRSV:SMN, AdRSV:Bcl-xL and AdRSV:Empty transduced SH-SY5Y cells treated with LY294002 (25μM).

Figure 7 A time-dependent increase in α-fodrin degradation following LY294002 treatment of differentiated SH-SY5Y cells. A: Western blot analysis of AdRSV:Empty transduced SH-SY5Y cells exposed to LY294002 (25μM); B: Densitometric analysis of the 145/150kDa α-fodrin degradation product; C: Densitometric analysis of the 120kDa α-fodrin degradation product.

Figure 8 Comparison of α-fodin degradation patterns following over-expression of SMN and Bcl-xL proteins. A: Western blot analysis of α-fodrin in differentiated SH-SY5Y cells transduced with AdRSV:SMN, AdRSV:Bcl-xL and AdRSV:Empty and treated with LY294002 (2h, 25μM). B: Densitometric analysis of the 145/150kDa α-fodrin degradation product; C: Densitometric analysis of the 120kDa α-fodrin degradation product.

Figure 9 Comparison of the caspase-3 p29 subunit status in transduced differentiated SH-SY5Y cells. A: Western blot analysis of caspase-3 after SMN and Bcl-xL over-expression and exposure to LY294002 (2h; 25μM); B: Densitometric analysis of the caspase-3 p29 subunit.

Figure 1A

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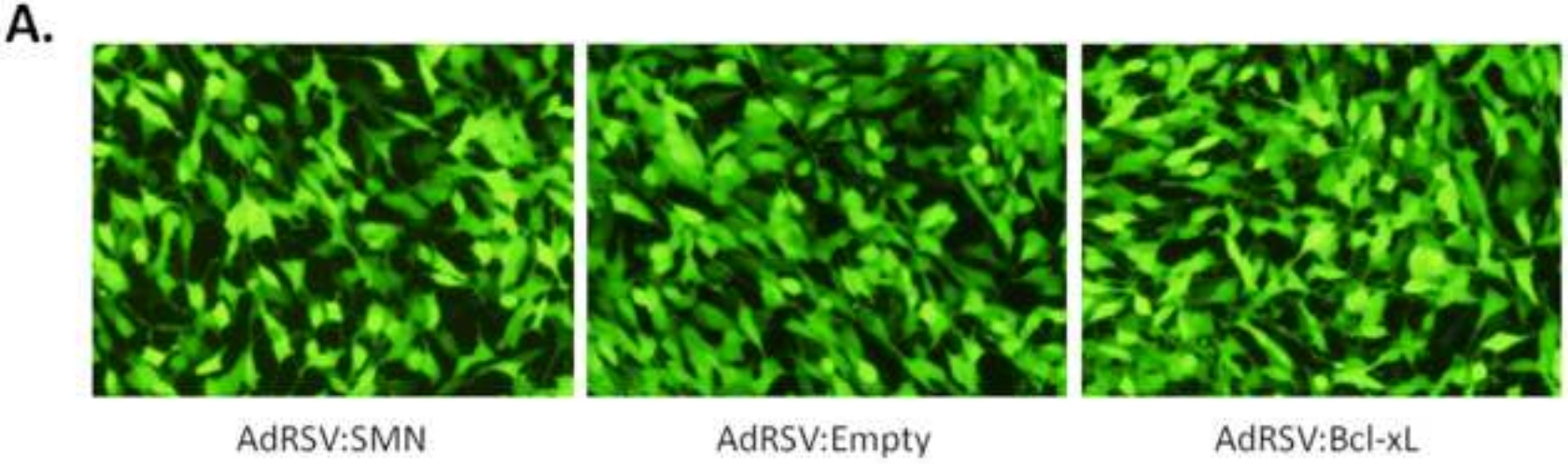

Figure 1B

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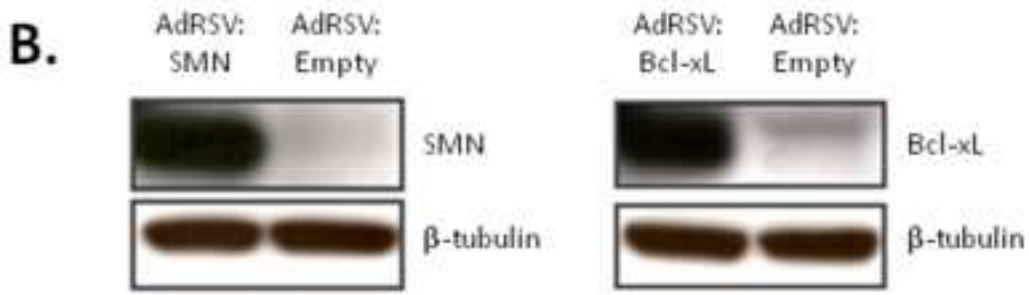

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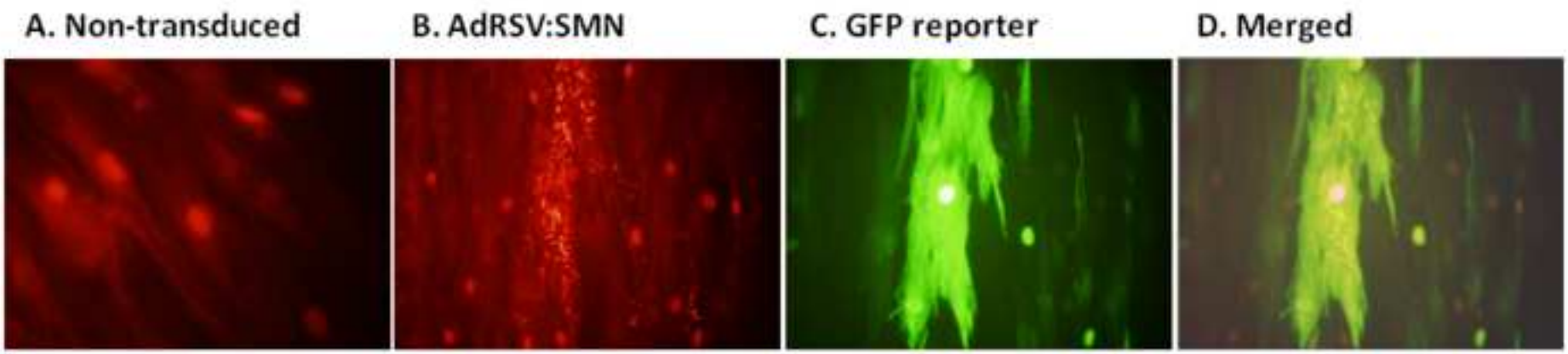

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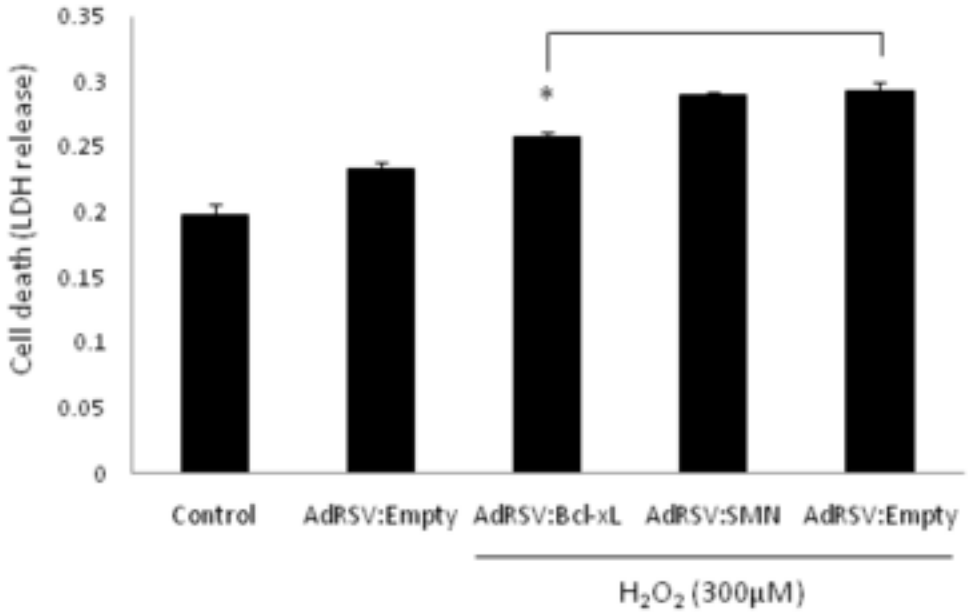

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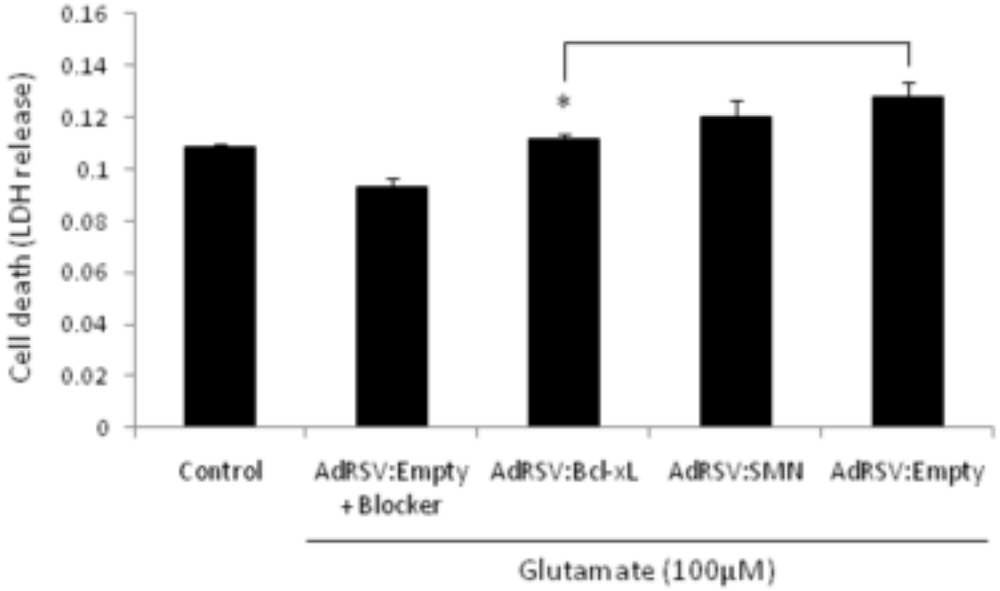

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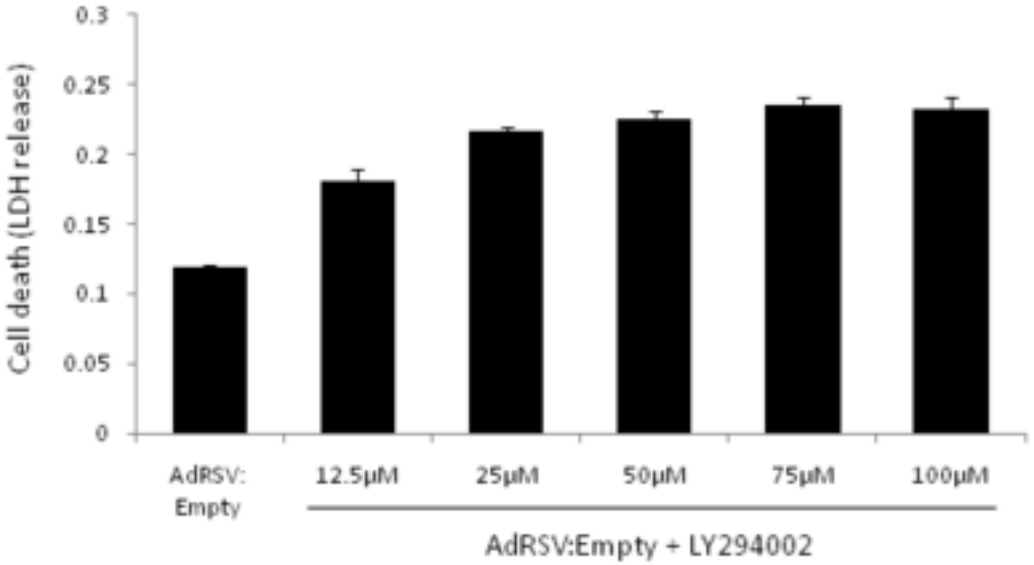

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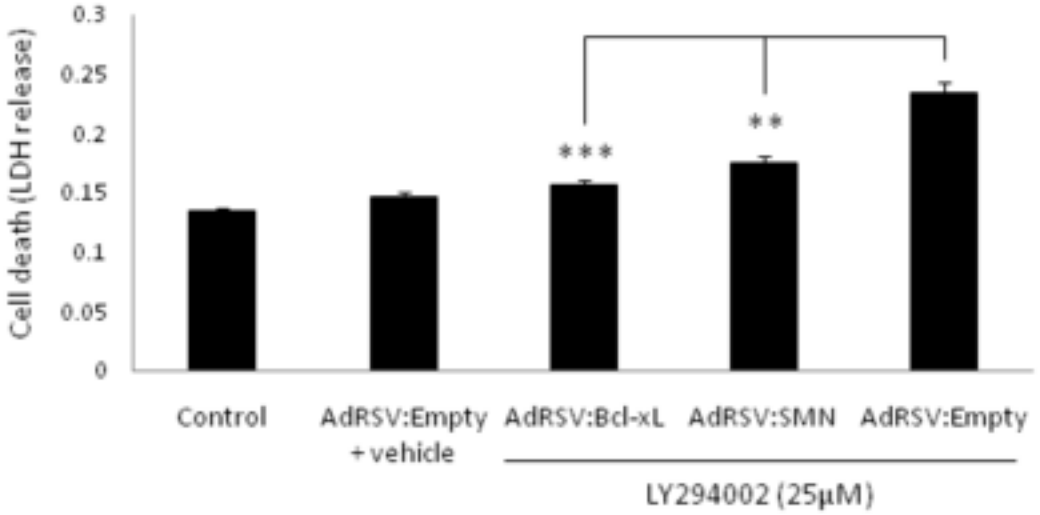

Figure 7A

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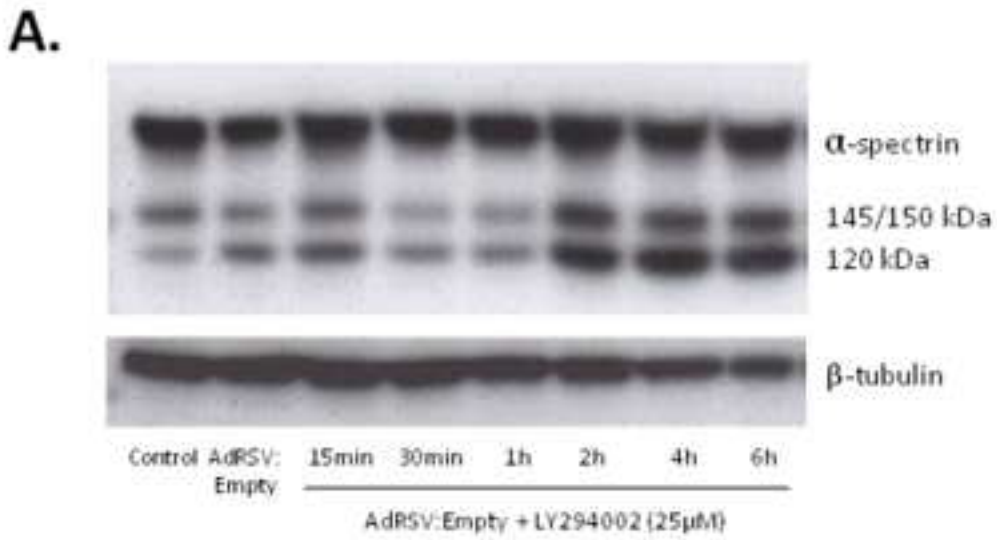

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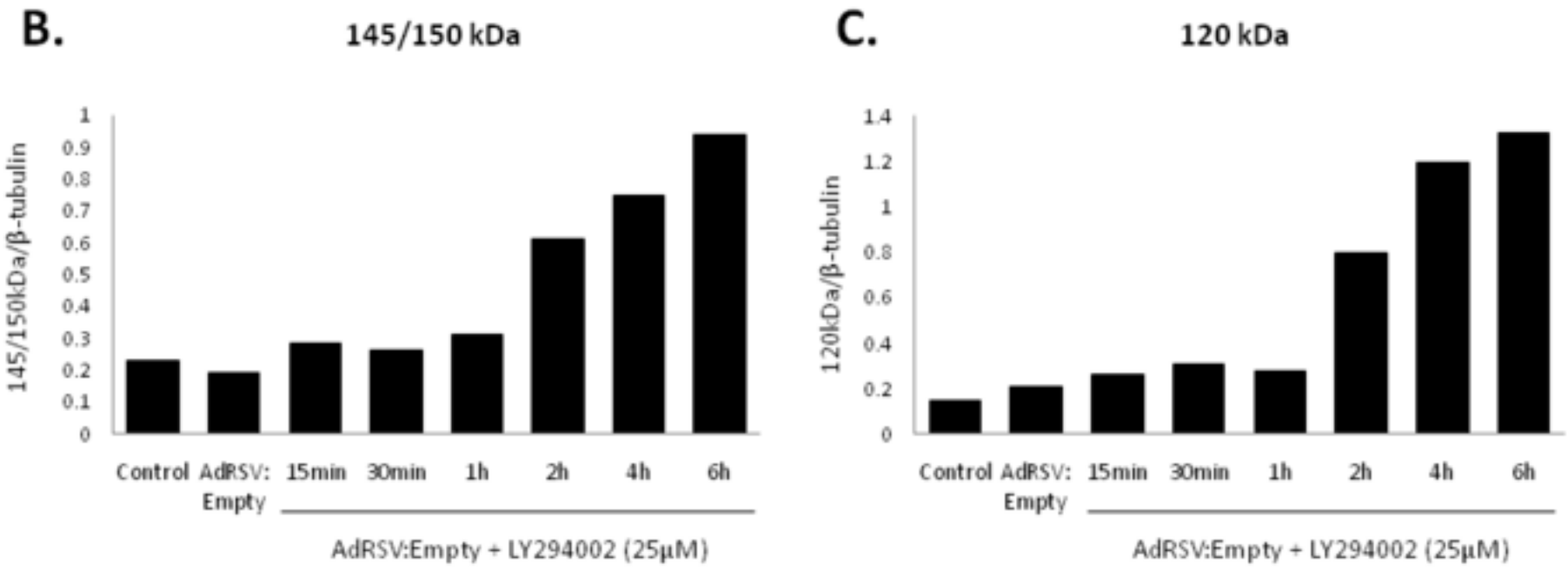

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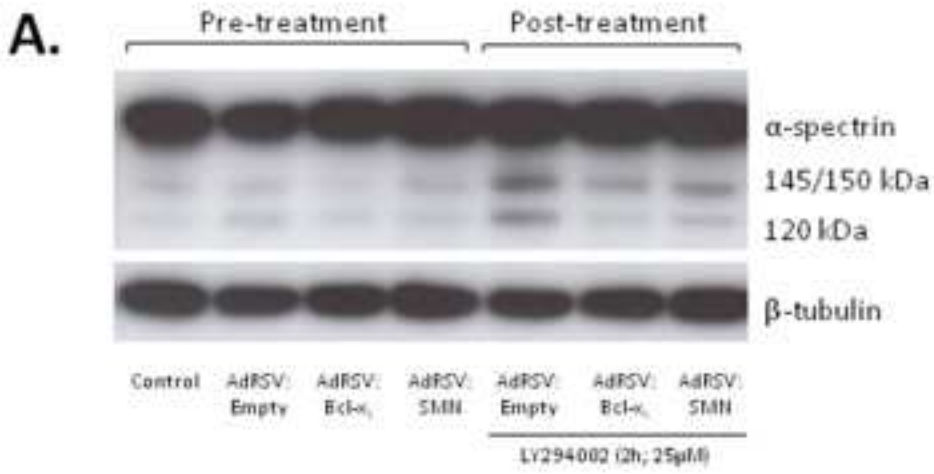

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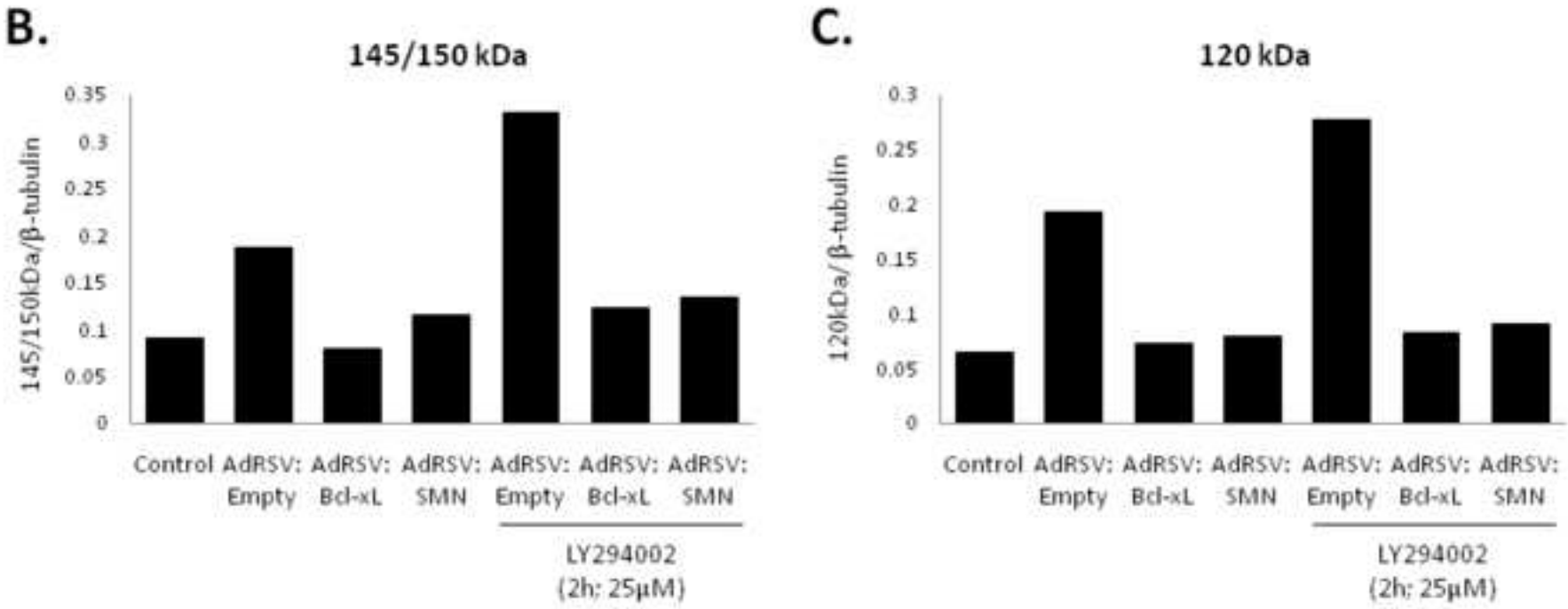

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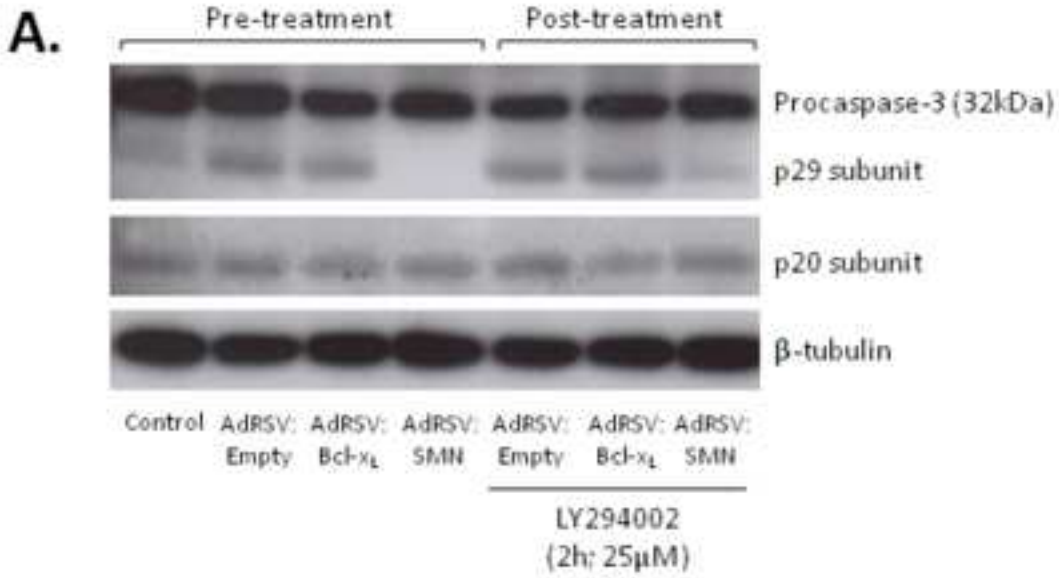

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